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TITLE: The Impact of 27-Hydroxycholesterol, a Macrophage-Produced Estrogen Receptor and Liver X Receptor Agonist, on Breast Cancer Pathophysiology

PRINCIPAL INVESTIGATOR: Donald P. McDonnell, Ph.D.

CONTRACTING ORGANIZATION: Duke University
Durham, NC 27708

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14. ABSTRACT We and others have recently demonstrated that 27-hydroxycholesterol (27HC) can act as a partial agonist for the estrogen receptors (ERs) and liver X receptors (LXRs). Macrophages are considered a major source of 27HC and macrophage infiltration is associated with more aggressive breast tumors. The goal of this study was to determine the impact of 27HC on breast cancer pathophysiology and evaluate the relative contributions of the ERs and LXRs in this regard. In this reporting period we show that LXR activation does not affect the basal proliferation rate of either ER positive or ER negative cell lines. However, LXR activation does decrease estradiol-mediated proliferation. LXR ligands alter the expression of known ER target genes in a differential manner. Macrophages secrete factors which alter the expression of known ER target genes, and we have confirmed that the products of CYP27A1 action are important among the macrophage secreted factors. In order to truly assess the consequences of macrophage secreted 27HC on breast tumor pathophysiology, we have generated genetic mouse models that will either have altered 27HC synthesis or metabolism. Finally, we show that elevation of 27HC increases primary tumor growth, decreases the time to a second detectable tumor, and increases the overall tumor burden of our model mice. In conclusion, we have made substantial progress on this project and believe that we are on schedule to complete the project on time. It is anticipated that at the conclusion of this study we will have a clear understanding of the role of 27HC on breast tumor pathophysiology.					
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Title: The Impact of 27-Hydroxycholesterol, a Macrophage-Produced Estrogen Receptor and Liver X Receptor Agonist, on Breast Cancer Pathophysiology.

Introduction:

In most estrogen receptor (ER) positive breast cancers, estrogens function as mitogens and thus, not surprisingly, antiestrogens and aromatase inhibitors have become frontline therapies in the management of this disease. However, for reasons that are not completely understood, most responsive tumors eventually relapse and manifest resistance to “anti-estrogen” therapies. Understanding the molecular mechanism(s) underlying resistance is therefore an issue of considerable practical importance. The mechanisms underlying acquired resistance remain unclear, and appear to be multi-factorial (for review see: 1). One of the most perplexing issues that has yet to be resolved is how tumors become resistant to aromatase inhibitors when the enzyme remains intact and when circulating levels of estradiol are undetectable. It is within the context of this observation, and the general problem of resistance to anti-hormone therapy as a whole, that we propose the hypothesis that breast tumors may be able to produce an atypical estrogenic ligand whose synthesis is not impacted by current interventions. In this regard, we and others have recently demonstrated that 27-hydroxycholesterol (27HC) acts as an agonist for both ER and liver X receptor (LXR) (2-5). These important findings, together with additional studies on the molecular pharmacology of this oxysterol, have led us to propose that 27HC may contribute to breast cancer pathology as; (1) it increases proliferation of breast cancer cells *in vitro*, (2) it is produced by macrophages, an increase in the number of which is associated with a poor prognosis in breast cancer, (3) outcome is poorer in patients whose tumors exhibit reduced expression of CYP7B1, the enzyme responsible for the metabolism of 27HC, in their primary tumors, and (4) 27HC induces the expression of key mitogens, chemotactic agents and angiogenic factors (such as stromal cell-derived factor 1 (SDF-1) and tumor necrosis alpha, (TNF)). Therefore, the main hypothesis being tested in this grant is that tumor associated macrophages contribute to ER positive breast cancer pathology by producing and secreting 27HC which then impacts the local tumor microenvironment. As detailed below, we have made significant progress in the last year, with Specific Aim 1 nearing completion and Specific Aims 2 and 3 well underway.

Body: (Months 1-12)

Specific Aim 1: Elucidation of the mechanism of action of 27HC in breast cancer.

Recent work by our lab and others has shown that 27HC is not only a ligand for the ERs, but also for the liver X receptors (LXR α/β) (3, 4, 6). Although the roles of LXR in breast cancer pathology are understudied, it has been shown *in vivo* that LXR activation increases the hepatic expression of estrogen sulfotransferase, which leads to increased E2 metabolism, decreased circulating E2 levels and decreased breast cancer xenograft growth (7). Furthermore, we have shown that ER can induce the expression of SHP, an LXR repressor highlighting how estrogens can abrogate the positive effects of LXR activation. Therefore we embarked on a series of experiments to characterize the impact of LXR activation on ER signaling and function in breast cancer.

We first investigated the effect of LXR activation by the synthetic LXR ligand, T0901317 (T1317) on breast cancer cell proliferation. Initially, MCF7 cells were chosen since they are ER α positive and have been shown in the past to be a good predictive model of antiestrogen/SERM activity *in vivo*. As expected, treatment with 17 β -estradiol (E2) significantly induced proliferation above that of vehicle treated cells (**Figure 1a**). 27HC treatment resulted in increased proliferation over vehicle, but only half that of E2 treated cells. T1317, at increasing concentrations from 10⁻⁷ to 10⁻⁵M, did not significantly alter proliferation from that of vehicle treated cells. However, T1317 ablated the E2 mediated increase in proliferation. Thus, although LXR activation had no significant effect on basal proliferation, it was capable of attenuating E2 stimulated proliferation.

One drawback to MCF7 cells is that they have a low basal proliferation rate in the absence of an estrogenic ligand, and thus it is difficult to assess whether LXR agonists were impacting proliferation *per se* or that induced by estrogen. Therefore, we performed similar proliferation experiments in two different ER α negative cell lines: SKBR3 cells (HER2 positive) and MDA-MB-231 triple negative (HER2-, PR- and ER α -). We found that increasing doses of the synthetic LXR ligands T1317 or GW3965 had no significant effect on the proliferation rate of either SKBR3 or MDA-MB-231 cells (**Figure 1b,c**). Additionally, 27HC did not alter the proliferation of these cells. Therefore, we conclude that LXR activation does not have a general inhibitory effect on proliferation but rather it has a specific effect on estrogen-dependent proliferation. Thus, we conclude that although 27HC can function as a partial ER-agonist and stimulate MCF-7 cell proliferation this effect may be balanced by its ability to induce the antiproliferative activities of LXR (**Specific Aim 1**).

In order to define how LXR activation attenuates the E2-proliferative response, we first tested the hypothesis that LXR agonists could directly or indirectly inhibit the transcriptional activity of ER. We noted that the LXR agonists, 27HC, T1317 and GW3965 all increased the transcription of ABCA1, a known LXR target gene (**Figure 2**). Notably, it was determined in multiple independent experiments in estrogen naive cells, that LXR activation (a) decreased the expression of BRCA1 and Ret, (b) modestly induced the expression of PS2 and PR, and (c) had no significant effect on SDK1 or WISP2 expression (**Figure 2, Experiment 1.1 - Task 1**). Whereas a comprehensive analysis of the impact of LXR activation on the ER/estradiol

transcriptome is underway, we have from our preliminary studies observed that LXR agonists were able to attenuate the E2 induction of BRCA1 and Ret and SDK1 but not PS2, PR or WISP2. Therefore, while direct LXR-dependent inhibition of ER target genes may contribute to the antiproliferative actions of LXR agonists, the mechanisms by which these two signaling pathways converge are likely to be more complex. These findings highlight how difficult it is to define the complex pharmacology of 27HC as it regulates the activities of two different receptors with opposing activities.

In the original grant proposal we hypothesized that 27HC, secreted by tumor associated macrophages, may act in a paracrine manner to affect breast cancer cell biology, or in an autocrine manner by inducing the synthesis of additional macrophage secreted factors (such as SDF-1 and/or TNF α) which then act on breast cancer cells to increase proliferation and/or invasiveness. We have now optimized techniques to differentiate macrophages from primary bone marrow derived monocytes *in vitro*, and have initiated studies to evaluate their ability to produce and respond to SDF-1 and TNF α and define how this is modulated by 27HC, estradiol and synthetic LXR ligands. (**Experiment 1.1 - Task 1**).

It has become clear that 27HC can act directly on ER α + breast cancer cells (**Figure 1a and 2** and (8)). Less clear, however, is whether the amount of 27HC synthesized by macrophages is sufficient to impact breast cancer biology. To address this issue, we made use of macrophages derived from wild type mice (CYP27A1+/+) or from mice that lack the capacity to synthesize 27HC (CYP27A1-/-). Spent media from these macrophages was added to MCF7 breast cancer cells and the expression of known ER α target genes was determined. ABCA1 was induced by spent media from wild type macrophages, and to a lesser extent by media from CYP27A1-/- macrophages (**Figure 3**). The ER responsive genes BRCA1, Ret, PS2, PR, SDK1, and WISP2 showed a similar trend in that their expression was induced by media from macrophages derived from wild type mice but minimally by media from CYP27A1-/- macrophages. Collectively, the data obtained from these experiments indicates that macrophages produce 27HC which can then act in a paracrine manner on breast cancer cells to stimulate the transcription of ER-dependent genes (**Experiment 1.2 – Task 1**). Linking these transcriptional responses to process of pathological importance in breast cancer is the next goal of these studies.

→Therefore, we fully expect to complete the objectives set out in Specific Aim 1 by the end of this granting period.

Specific Aim 2: Definition of the role of 27HC in the pathophysiology of breast cancer, *in vivo*.

As outlined in the grant proposal, it was necessary to establish a model system(s) that would allow for the evaluation of the impact of tumor-associated macrophages on the pathophysiology of breast cancer. To accomplish this, we have chosen to use a mouse model of mammary carcinoma (polyoma middle T, PyMT) (9). These mice carry the MMTV-PyVT transgene, develop spontaneous adenocarcinomas of mammary epithelium by 8 weeks of age, and have been validated as an appropriate model to investigate the multistep progression of breast cancer. Furthermore, the tumors that arise in this model are estrogen responsive and

readily metastasize to the lung (10-12). To test the hypothesis that 27HC impacts breast cancer pathophysiology we proposed two different approaches. Firstly, using genetic knockout (CYP27A1^{-/-} and CYP7B1^{-/-}) the impact of 27HC on the development and progression of tumors will be assessed. Secondly, we proposed to elevate 27HC by injection from the time a palpable tumor is found. We have now received IACUC and ACURO approval for all of our intended animal studies (**Milestone 1**).

(A) Experiment 2.1: Breast cancer in mouse knockout models.

The studies proposed in the first experiment required us to establish a breeding program to generate mouse models where the gene that synthesizes 27HC (CYP27A1) or that responsible for its metabolism (CYP7B1) has been knocked out. Since CYP27A1^{-/-} mice and CYP7B1^{-/-} are maintained on different strains, we had to generate four new mouse models: (1) CYP27A1^{+/+}, PyMT⁺, (2) CYP27A1^{-/-}, PyMT⁺, (3) CYP7B1^{+/+}, PyMT⁺, and (4) CYP7B1^{-/-}, PyMT⁺. We obtained male PyMT⁺ mice from Jackson Labs (**Task 1**), and implemented a breeding scheme to obtain mice with the required genotypes. We have also optimized DNA genotyping protocols for these studies (**Task 2-3**). Recently, we have obtained our first experimental animals and are monitoring their tumor growth (**Task 4-5**). Tasks 4 and 5 are ongoing as new mice are born. Therefore, we are on schedule to accomplish the objectives of this experiment outlined in the initial proposal.

(B) Experiment 2.2: Direct effects of 27HC on breast cancer, *in vivo*.

The impact of exogenous 27HC on tumor growth was next assessed to define its specific role in tumor pathology. For these studies we obtained male PyMT⁺ (otherwise wild type) mice from Jackson Labs (**Task 1**). A program where PyMT⁺ males are bred to PyMT⁻ females has been established to generate PyMT⁺ females for use in injection studies (**Task 2**). In order to eliminate the confounding effects of estrogen, we have used ovariectomized females for our experiments. Injection studies with placebo and 27HC are now underway (**Task 3**). Based on our preliminary results, it appears that primary tumors grown in mice treated with 27HC grow faster in the initial stages of growth compared to those in placebo treated mice (**Figure 4**). Intriguingly, between 28 and 32 days after its first detection the growth rate of tumors treated with 27HC slowed compared to those treated with placebo. It is possible that this slowing may be due to the loss of the ER, leaving 27HC to work only through the ‘protective’ LXRs; a hypothesis which is now being addressed in **Specific Aim 3**. Additionally, we noted that 27HC treatment decreases the time period from the onset of the primary tumor to the detection of a second palpable tumor (**Figure 5**). This decreased time is likely due to a 27HC mediated increase in the growth rate of secondary tumors since it is apparent from **Figure 4** that 27HC increases the growth rate at early stages. This is clearly reflected when measuring the total tumor burden (**Figure 6**); 27HC treated mice have an increased total tumor burden. Collectively, our preliminary data lends strong support to our hypothesis that 27HC impacts the growth of mammary tumors.

Specific Aim 3: Defining the roles of ER α and the LXRs in 27HC modulated breast cancer pathology.

We have acquired IACUC and ACURO approval for the studies in this aim (**Milestone 2**).

(A) Experiment 3.1: The ER mediated effects of 27HC on breast cancer.

We originally proposed to address this issue by crossbreeding PyMT mice onto an LXR α/β double knockout strain, and subsequently treating with 27HC. Unexpectedly, however, we have had problems generating the LXR α/β double knockout strain and the poor yield in animals with the required genotypes makes this approach unfeasible. Therefore, we propose to compare the effects of the pure ER ligand 17 β -estradiol to those of 27HC in PyMT mice. To this end, we have achieved ACURO approval and have initiated a breeding program to obtain experimental animals for treatment.

(B) Experiment 3.2: The LXR mediated effects of 27HC on breast cancer.

We are currently breeding mice for these studies (**Task 2**). Furthermore, we propose to determine the direct effects of LXRs on tumor growth *in vivo* by treating with a synthetic LXR agonist, GW3965. By comparing the results of 27HC treatment to those of GW3965 treatment, we will be able to determine relative effects (if any) of 27HC mediated through the LXRs.

Key Research Accomplishments:

We have now completed an important series of *in vitro* studies the combined results of which support our hypothesis that 27HC, acting through ER and LXR, implicate this oxysterol in breast cancer pathophysiology . Our *in vivo* studies clearly indicate that 27HC impacts breast cancer pathology and we are in the process of breeding animal models which will be used to further characterize the roles of macrophage-secreted 27HC.

- Our data indicate that LXR activation does not affect the basal proliferation of breast cancer cells.
- LXR activation does however attenuate the E2-stimulated proliferation in MCF7 cells.
- LXR activation decreases the expression of some known ER target genes, has no effect on some, or increases others, implicating a potentially complicated cross-talk between ER and LXR signaling.
- Macrophages secrete factors that increase the gene expression of the LXR target gene, ABCA1 and various ER target genes. We have confirmed that the products of CYP27A1 are important among the macrophage secreted factors.
- We have established a breeding colony of mice carrying the PyMT transgene.
- Crossbreeding has resulted in the development of the following mouse models:
 - CYP27A1^{+/+}, PyMT⁺
 - CYP27A1^{-/-}, PyMT⁺
 - CYP7B1^{+/+}, PyMT⁺
 - CYP7B1^{-/-}, PyMT⁺
- Elevated 27HC increases the initial growth phase of breast tumors in PyMT mice.
- 27HC treatment decreases the time to secondary tumor detection and thus increases the growth of total tumor burden.

Reportable Outcomes:

- Crossbreeding has resulted in the development of the following mouse models:
 - CYP27A1^{+/+}, PyMT⁺
 - CYP27A1^{-/-}, PyMT⁺
 - CYP7B1^{+/+}, PyMT⁺
 - CYP7B1^{-/-}, PyMT⁺

Conclusion:

Our current research is directed towards an evaluation of the potential impact of 27HC secreted by tissue associated macrophages in breast cancer pathology. Both *in vitro* and *in vivo* approaches are being employed to determine specific versus tumor microenvironment effects. Our primary hypothesis is that macrophages contribute to ER α + breast cancer pathology by producing or secreting 27HC and impacting the local tumor microenvironment. Our *in vitro* studies are aimed at (a) defining the roles of the LXRs in breast cancer biology, and (b) providing evidence that 27HC, synthesized and secreted from macrophages, can significantly impact breast cancer. In order to specifically define the role(s) of macrophage derived 27HC on breast tumor pathophysiology, we have generated genetic mouse models that exhibit altered 27HC synthesis and/or metabolism. Elevating 27HC by injection in PyMT mice, a model that spontaneously develop breast cancer, results in a primary tumor that grows faster than in placebo treated mice. Furthermore, the time to detection of a secondary tumor is reduced in 27HC treated mice with a corresponding increase in the total tumor burden (summed volume of all palpable tumors). In conclusion, we have made substantial progress on this project and believe that we are on schedule to complete the project on time. It is anticipated that at the conclusion of this study we will have a clear understanding of the role of 27HC on breast tumor pathophysiology.

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Appendix 1: Figures

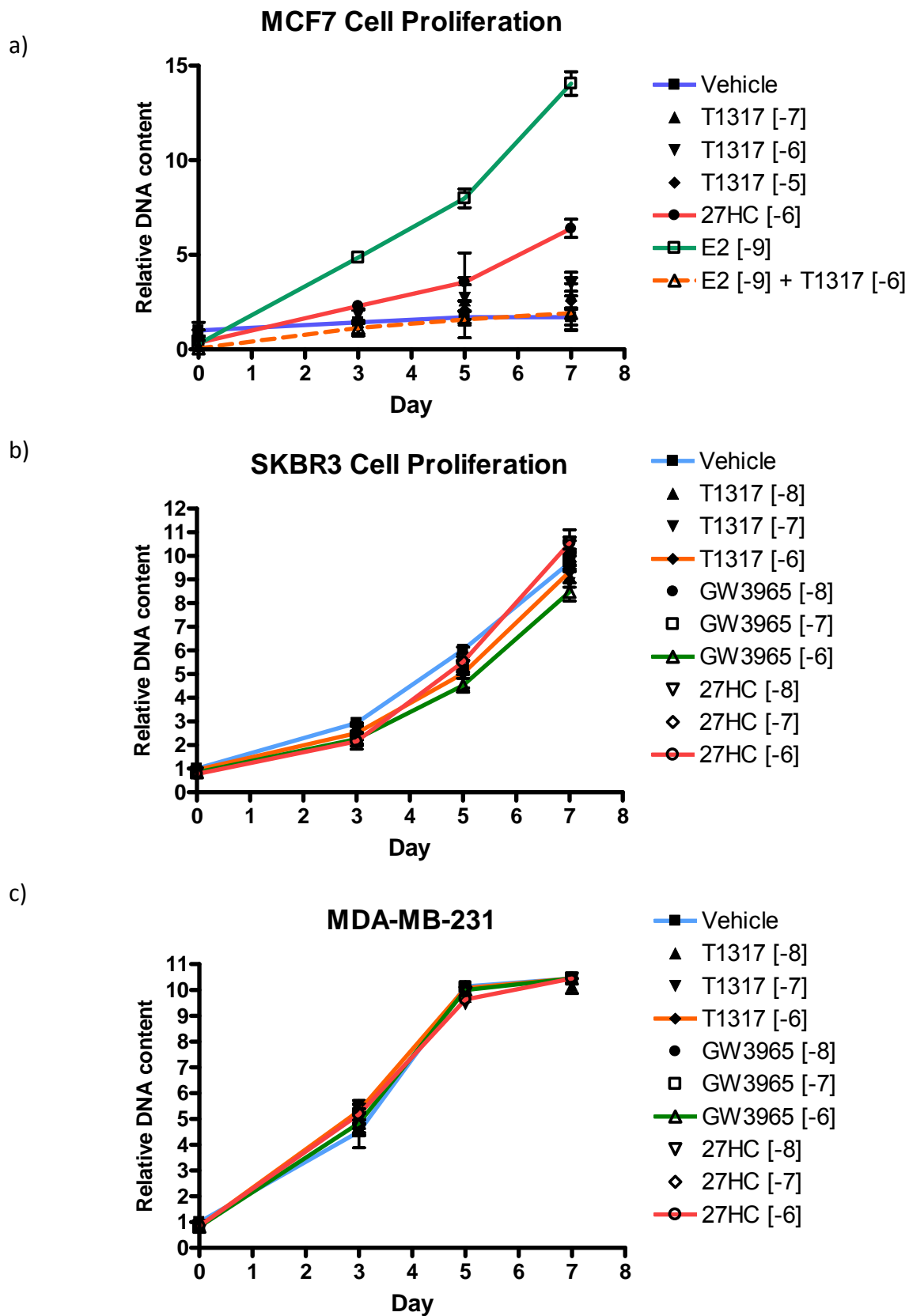


Figure 1: Effects of 27HC and synthetic LXR agonists on cell proliferation in a) ER α + MCF7 cells, b) ER α -, HER2+ SKBR3 cells, or c) ER α -, PR- and HER2- MDA-MB-231 cells.

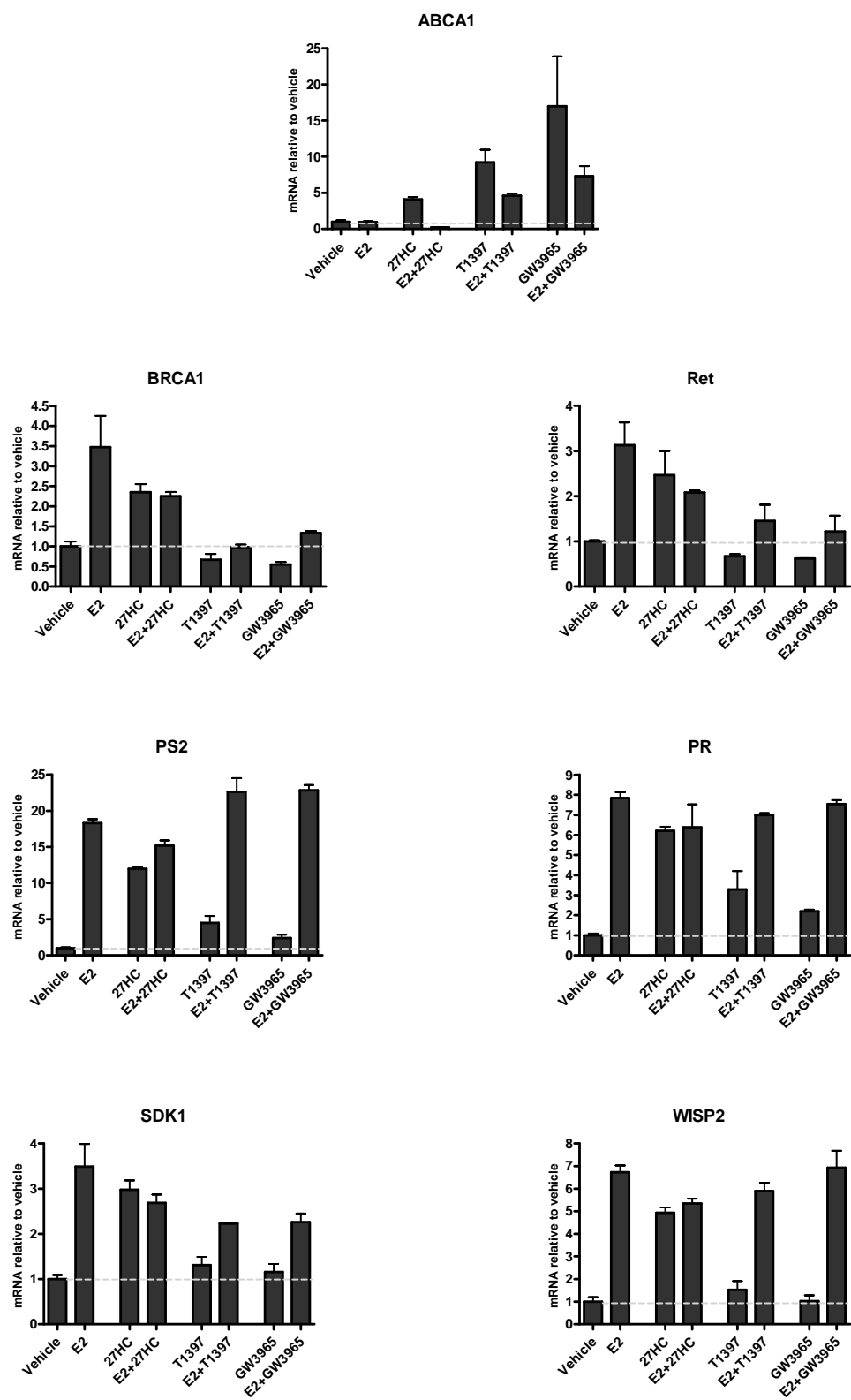


Figure 2: Effects of LXR agonists on E2 mediated gene transcription in MCF7 cells.

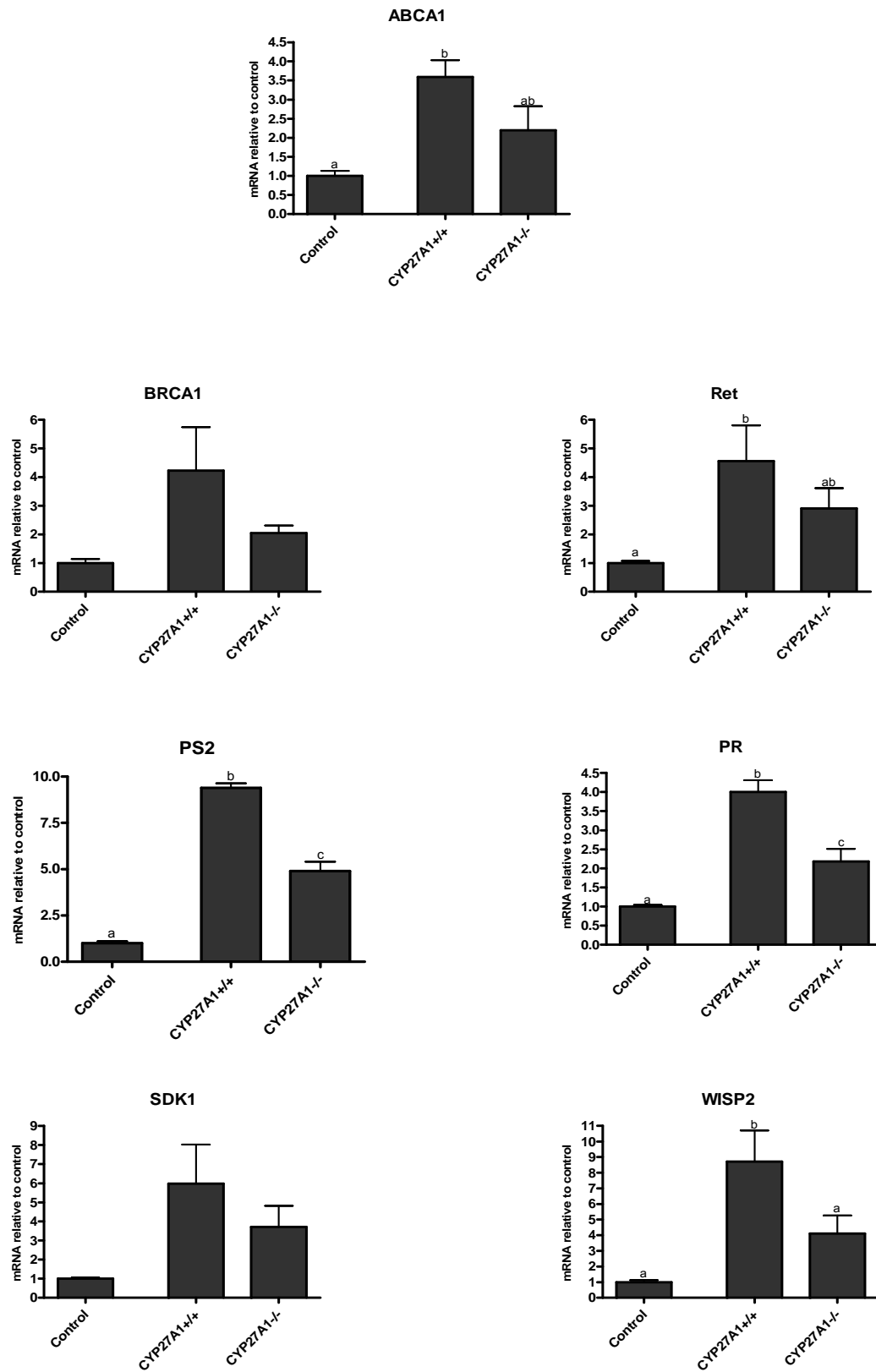


Figure 3: Effects of spent macrophage media on gene expression in MCF7 cells.

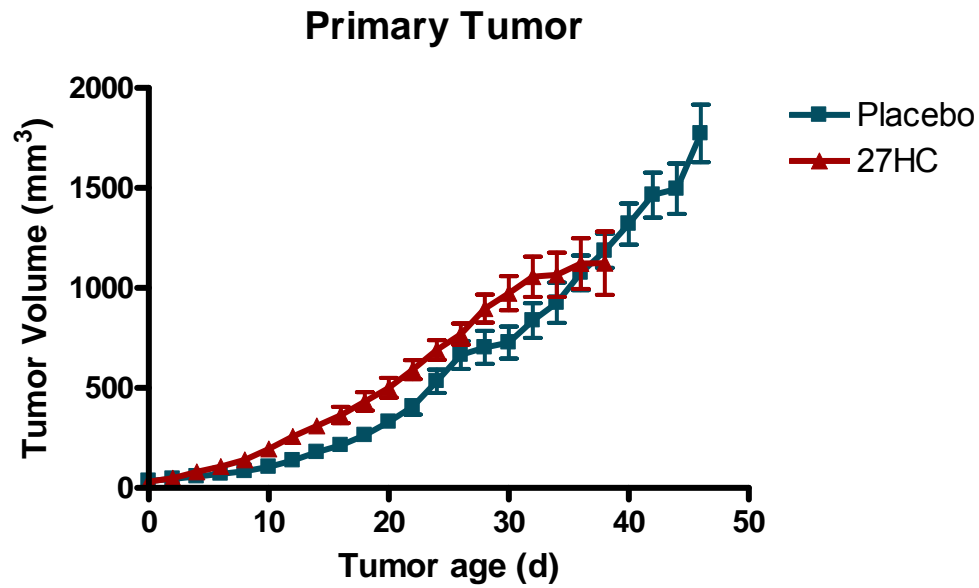


Figure 4: Effect of 27HC on primary tumor growth in PyMT transgene expressing mice. Mice were ovariectomized between 5 and 6 weeks of age. Upon detection of a palpable mammary tumor, mice were treated daily with a subcutaneous injection of placebo or 27HC. Tumor growth was assessed by direct caliper measurement.

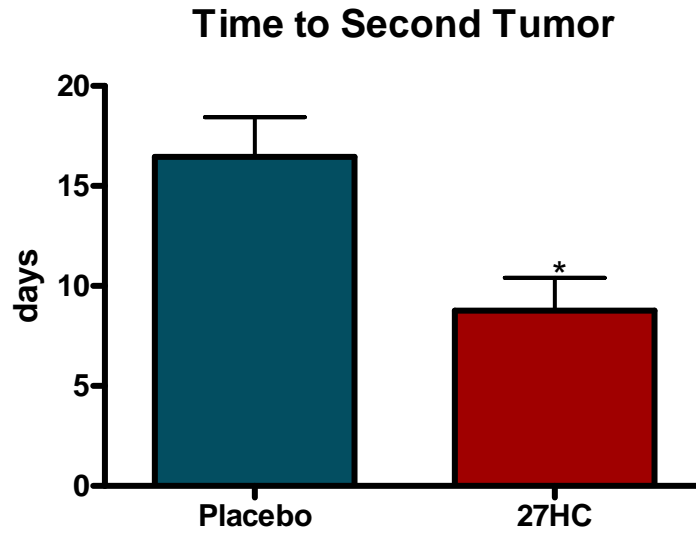


Figure 5: Treatment 27HC decreases the time between onset of primary tumor and detection of a secondary tumor by palpation. See Figure 4 for details. Star represents statistically significant difference (unpaired t-test, $P < 0.01$).

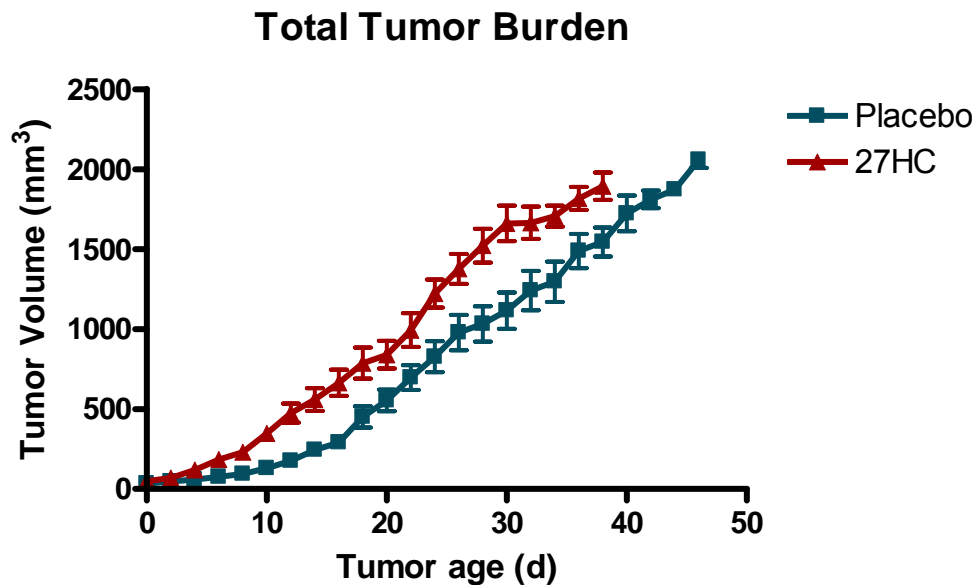


Figure 6: 27HC increases total tumor burden in PyMT mice. Total tumor burden was determined by adding the volumes of all individual tumors. See Figure 4 for details.